TRITIUM KINETIC ISOTOPE EFFECTS ON ENZYMATIC DECOMPOSITION OF 5'-HYDROXY-L-TRYPTOPHAN

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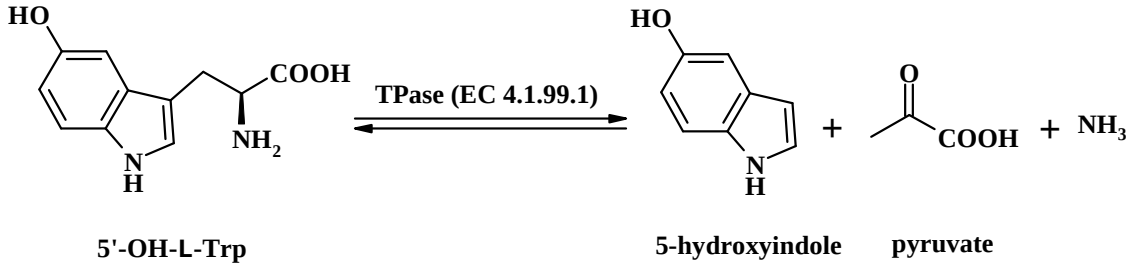
Abstract

The tritium kinetic isotope effect on position 2 has been determined in the reaction of decomposition of 5'-hydroxy-L-tryptophan, 5'-OH-L-Trp, catalysed by enzyme TPase, (EC. 4.1.99.1). The numerical values of isotope effects in the course of reaction were obtained by competitive method using 5'-hydroxy-[1-^{14}C]-L-tryptophan as internal radiometric standard.

Introduction

The enzyme tryptophanase (L-Tryptophan indole lyase EC 4.1.99.1), TPase, catalyses the decomposition of 5'-OH-L-Trp to the corresponding 5-hydroxyindole, pyruvic acid, and ammonia [1]. (Scheme 1).



Scheme 1. The reversible reaction catalyzed by the enzyme Tpase

Under certain conditions, there is a possibility of the reverse reaction, leading to formation of L-tryptophan. This enzyme also decomposes L-serine, L-cysteine, S-methyl-L-cysteine, and is often used in synthesis of 5'-hydroxy-L-tryptophan [2]. In the literature, the mechanism of TPase catalysis are well documented [3, 4]. One technique often used in studying such kind of reaction mechanisms is kinetic isotopic effect method (KIE). In the course of KIE study the ratios of reaction rates for lighter and heavier isotopes should be determined since numerical values of KIE's will be used to find rate determining step.

Aforementioned studies require the synthesis of isotopomer of 5'-OH-L-Trp labeled with isotopes of hydrogen in 2-position of side chain and isotopomer of this compound specifically labeled with ^{14}C in carboxylic group. For measuring reliable values of KIE we applied internal radioactive standard method. It assumes using doubly labeled substrate for KIE determination. The substrate, in our case 5'-OH-L-Trp, should bear one label (^{14}C) in position non-involved in course of reaction, and second label

(tritium) is bonded to 2-position. In this method KIE is determined from changing the value of ratio $^{14}C/^{3}H$ in course of reaction.

For this studies we have used the following isotopomers: 5'-OH-[2-^{3}H]-L-Trp and 5'-OH-[1-^{14}C]-L-Trp. The ^{14}C-labelled compounds were used as internal radiometric standards for precise determination of numerical values of KIE.

The synthesis of abovementioned isotopomers of tryptophan have been described by us earlier [5, 6].

Results and discussion

Tritium kinetic isotope effects ($^{1}H/^{3}H$) of hydrogen bonded to α-carbon position of 5'-OH-L-Trp in course of decomposition have been measured in water in room temperature. The values of tritium kinetic isotope effects in mentioned above reaction are given in Table 1.

Table 1. Tritium KIE for enzymatic decomposition of 5'-OH-[2-^{3}H]-L-Trp

Degree of conversion [f]	KIE
0,022	4,09
0,061	4,99
0,063	4,62
0,100	4,48
0,107	4,87
0,142	4,49
0,142	5,00
0,163	4,56
0,180	4,79
0,220	4,43
	Avg. 4,63 ± 0,16

These effects were determined using internal radiometric standard ([1-^{14}C]-5'-OH-L-Trp) and Yankwich-Tong equation (1) to calculate KIE values [7]. Internal radioactive standard method assumes using $^{3}H/^{14}C$ ratio instead of specific activity of ^{3}H-labeled 5'-OH-L-Trp, therefore, the determination of KIE is much more precise. The experimental error was accessed by Student t-test with 95% of confidence.

$$\alpha = \frac{\ln[1 - f\,\frac{(1-f)R_0}{R_f}]}{\ln(1-f)} \qquad (1)$$

where:

- α - $^{1}H/^{3}H$ kinetic isotope effect,
- R_0 - $^{3}H/^{14}C$ radioactivity ratio in 5'-OH-L-Trp at the start of reaction,
- R_f - $^{3}H/^{14}C$ radioactivity ratio in 5'-OH-L-Trp after f degree of conversion,
- f - degree of conversion.

Considerably large KIE of tritium implies that it value is typical to primary KIE, and therefore, the hydrogen atom in position 2 plays a significant role in transformation of the enzyme-substrate complex into enzyme-product complex. In this study, the kinetic isotope effect of enzymatic decomposition of 5'-OH-L-Trp was determined for the first time using the radioactive isotopes. While, KIE for this reaction has been previously investigated, it was relied upon the stable isotopes, specifically determining the solvent isotope effects. The magnitude of KIE for tritium indicates that hydrogen atom bonded with α-carbon of 5'-OH-L-Trp is involved in a proton transfer during the decomposition of a tryptophan.

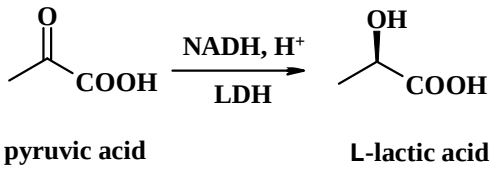
Experimental

Materials

All chemicals was from Aldrich. Enzymes TPase (EC 4.1.99.1) from *Escherichia coli*, LDH (EC 1.1.1.27) from rabbit muscle, cofactor PLP, and NADH were from Sigma. The scintillation cocktail was from Rotiszint (Germany).

Methods

The concentration of pyruvic acid was determined spectrophotometrically using Shimadzu UV-102 CE-LV spectrometer. This indirectly procedure consists of conversion of pyruvic acid to L-lactic acid by enzyme LDH and coenzyme NADH (Scheme 2) and determining the concentration changes of NADH by measuring the absorbance at 340 nm [8, 9].



Scheme 2. Conversion of pyruvic acid into L-lactic acid by enzyme LDH

The radioactivity of all samples was determined using an automatic liquid scintillation counter (LISA LSC PW470 – Raytest, Germany).

KIE assays

KIE assays was carried out at room temperature. In catalysed reaction by TPase the LDH/NADH couple allows indirectly determine the concentration of pyruvic acid [11] (scheme 2), and degree of decomposition of 5'-OH-L-Trp.

For each kinetic run the assayed sample of [2-^{3}H]-, and [1-^{14}C]-5'-OH-L-Trp was placed in encapped vial and dissolved in 4.5 ml of 0.1 M phosphate buffer, pH 8. Radioactivity of ^{3}H-isotopomer was 3-7 fold higher than ^{14}C-one. To this the following reagents were added in turn:

1. 90 mg of KCl;
2. 9,5 mg D,L-dithiothreitol ($HSCH_2(OH)CH_2(OH)CH_2SH$ 1,4-dithiobutan-2,3-diol,);
3. 300 l of 1mM 5'-pyridoxal phosphate, PLP, (cofactor);
4. 600 l of the enzyme L-lactic dehydrogenase of activity 270 U/ml;
5. 300 l of 2 mM NADH;
6. 300 l of enzyme TPase (0.13 U/ml).

In the preset time of the course of reaction the 1 ml of volume samples were taken, and degree of conversion was determined spectrophotometrically. Next, reaction was quenched by acidifying the reaction mixture to pH 5 with glacial acetic acid. The non reacted 5'-OH-L-Trp and L-lactic acid were separated on ion exchange column (Amberlite IR 120 H^{+} form, 60×5 mm) by elution with 0.3 M NH_3(aq) and their radioactivities were measured on LSC. Additionally the degree of conversion was checked using radioactivity of the product and substrate.

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